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(54) Title: HUMAN OVARIAN TUMOR-ASSOCIATED ANTIGEN SPECIFIC FOR MONOCLONAL ANTIBODY OV-TL3

(57) Abstract

An antigen associated with ovarian carcinoma is described. The antigen is expressed by ovarian carcinoma of the serous, mucinous, endometrioid and clear cell types. Methods of diagnosis and therapy of ovarian carcinoma based upon the discovery and characterization of the antigen are also described.

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Human ovarian tumor-associated antigen specific for monoclonal antibody OV-TL3.

Background of the Invention

Ovarian cancer is the most lethal of all gynaecological cancers. Most of the ovarian tumors are of the epithelial type, of which the serous and mucinous cystadenocarcinomas predominate. The detection of ovarian tumors is often fatally delayed because of their location and the lack of diagnostic methods for early detection. By the time most patients are diagnosed they have advanced ovarian carcinoma (FIGO Stage III or IV), and their prognosis is poor. Attempts have been made to identify tumor markers which would allow both an early diagnosis and treatment, thus improving the prognosis for patients. See e.g., Lloyd, K.O.: Human ovarian tumor antigens. In: Serological Analysis of Human Cancer Antigens, Rosenberg, S.A. (ed.), New York, Academic Press 1980, pages 515-524; Bhattachary M. and Barlow, J.J.: Tumor markers for ovarian cancer, Int. Adv. Surg. Oncol. 2:155-176, 1979; Lloyd, K.O.: Human tumor antigens: Detection and characterization with monoclonal antibodies. In: Basic and Clinical Tumor Immunology; Huberman, R.B. (ed.), Martinus Nijhoff Publishers, Boston, 1983, pages 159-214. Cytological analysis of serous effusions for malignant cells, at a late stage of the disease, is conclusive in the majority of samples. However, in approximately 15% of the samples a definite diagnosis cannot be made due to

the similarity between well differentiated adenocarcinoma cells and reactive atypical mesothelial cells. (Epenetos, A.A., Canti, G., Taylor-Papadimitriou, J., Curling, M. and Boomer, W.F.: Use of two epithelium specific monoclonal antibodies for diagnosis of malignancy in serous effusions, The Lancet ii: 1004-1006, 1982).

The identification of tumor-associated antigen markers has been shown to be an important tool for diagnosis and surveillance of women with ovarian cancer. It was the development of the monoclonal antibody technique which facilitated the search for phenotypic heterogeneity of tumors and normal tissues and the recognition of these tumor-associated antigens. (Bhattacharya, M., Chatterjee, S.K., Barlow, J.J. and Fuji, H., Cancer Research, 42:1650-1654, 1982; Bast, R.C., Feeney, J. et al., J. Clin. Invest., 68:1331-1337, 1981; Borowitz, M.J. and Stein, R.B., Arch. Pathol. Lab. Med., 108:101-105, 1984). Monoclonal antibodies can be used as targets for attacking cancer cells for both diagnosis and treatment of cancer.

Since the original description of monoclonal antibody OC125 (Bast, R.C., Feeney, M. et al., J. Clin. Invest., 68:1331-1337, 1981) several other monoclonal antibodies have been reported which react with human ovarian carcinomas (Mattes, J.M. et al., Proc. Natl. Acad. Sci. 81:568-572, 1984; Tabliabue, A. et al., Cancer Res. 45:379-385, 1985; Tsuji, Y. et al. Cancer Res. 45: 2358-2362, 1985; Thor, A. et

al., J.N.I.C. 76:995-1006, 1986; Poels, L.G. et al., J.N.C.I. 75:781-791, 1986; Taylor-Papadimitriou, J. et al. , Int. J. Cancer 28:17-21, 1981.); these antibodies have been used to monitor the disease (Bast, R.C. et al., N. Eng. J. Med. 309:883-887, 1983), to detect tumors by means of radionuclide imaging (Epenetos, A.A. et al., Cancer 55:984-987, 1985; Pateisky, N. et al., J. Nucl. Med. 26:1369-1376, 1985) and to treat tumors by radioimmunotherapy (Epenetos, A.A. et al., Obstet. Gynecol. 68:715-745, 1986). However, the lack of sufficient tumor specificity is still a problem. In the adult, OC125 defined antigen can also be detected in the lining epithelium of the gynaecological tract. Elevated CA125 tumor antigen levels (65U/ml) were found in serum from 22% of patients with non-gynaecological cancers, including carcinomas of the pancreas, stomach, colon and breast. But also a limited number of false positives could not be excluded entirely in the same study. (Bast, R.C. and Klug, T.L. et al., Amer. J. Obstet. Gynaecol., 149(5):553-559, 1984.)

Summary of the Invention

This invention relates to a cell surface antigen (designated CA-TL3) associated with primary and metastatic human ovarian carcinoma of the serous, mucinous, endometrioid and clear cell types which demonstrates specific reactivity with the OV-TL3 monoclonal antibody. The invention also

relates to methods of diagnosis and therapy of ovarian carcinoma which are based upon the CA-TL3 antigen as a distinctive marker of the tumor.

The CA-TL3 antigen is a cell surface antigen expressed by the major histological types of ovarian carcinoma. The antigen is not expressed by non-gynaecological tumors and is only minimally expressed by normal epithelium of the female genital tract.

Because of its association with ovarian carcinoma, the CA-TL3 antigen can be used to diagnose and treat ovarian carcinoma. For example, monoclonal antibodies which are specific for the CA-TL3 antigen can be produced and used for the detection, diagnosis and treatment of ovarian carcinoma based upon their reactivity with CA-TL3. These monoclonal antibodies can be used to image primary and/or metastatic ovarian carcinoma in vivo by immunoscintigraphy, thereby allowing tumor localization. In therapy, these antibodies may be used for passive immunotherapy or as a targeting agent for selective delivery of cytotoxic agents to ovarian carcinoma in vivo.

Brief Description of the Drawings

Figure 1A is a photograph of an immunoblot from a 10% SDS-polyacrylamide gel of OVCAR-3 cell lysates probed with radioiodinated OC125.

Figure 1B is a photograph of an immunoblot from a 7.5% SDS-polyacrylamide gel of OVCAR-3 cell lysates probed with radioiodinated OV-TL3.

Figure 2 shows the double reciprocal plot of binding of antibodies to OVCAR-3 cells. (o=binding of ^{125}I-OC125, *=binding of ^{125}I-OV-TL3.)

Figure 3 shows the binding of the antibodies to OVCAR-3 cells over time.

Figure 3A depicts the binding of ^{125}I-OC125 to glutaraldehyde fixed cells (o) or non-fixed cells (*).

Figure 3B depicts the binding of ^{125}I-OV-TL3 to glutaraldehyde fixed cells (o) or non-fixed cells (*).

Figure 4 shows the dissociation of antibodies from OVCAR-3 cells over time.

Figure 4A depicts the binding of ^{125}I-OC125 to glutaraldehyde fixed cells (o) or non-fixed cells (*).

Figure 4B binding of ^{125}I-OV-TL3 to glutaraldehyde fixed cells (o) or non-fixed cells (*).

Figure 5 shows the inhibition of antibody binding by serum and ascites samples. (o=binding of ^{125}I-OC125, *=binding of ^{125}I-OV-TL3.)

Figure 6 shows the cross inhibition of the antibodies.

Figure 6A depicts the binding of ^{125}I-OC125 with different concentrations of inhibitor OC125 (o)

and with different concentrations of inhibitor OV-TL3 (*).

Figure 6B depicts the binding of ^{125}I-OV-TL3 with different concentrations of inhibitor OC125 (o) and with different concentrations of inhibitor OV-TL3 (*).

Figure 7 shows the biodistribution of the antibodies in OVCAR-3 bearing nude mice at (A) 24 and (B) 125 hours after intravenous administration of the antibodies. (speckled bars = ^{125}I-OC125, hatched bars = ^{125}I-OV-TL3.)

Detailed Description of the Invention

This invention relates to a cell surface antigen (designated CA-TL3) associated with primary and metastatic human ovarian carcinoma of the serous, mucinous, endometrioid and clear cell types (i.e. the major histological types of ovarian carcinoma). The CA-TL3 antigen is expressed by primary and metastatic ovarian tumor cells of these histological types. This antigen is further characterized by its specific reactivity with the OV-TL3 monoclonal antibody. The invention also relates to methods which employ monoclonal antibodies to diagnose and treat ovarian carcinoma based upon the use of CA-TL3 antigen as a distinctive marker of the tumor. The monoclonal antibodies OC125 and OV-TL3 were compared in terms of their interaction with ovarian carcinoma cells in

an attempt to further characterize the antigens recognized by these antibodies.

Some of the characteristics and properties of the antigen provided by the present invention include the following:

(a) Ovarian cancer cell lysate (OVCAR-3 cell line) antigens analyzed by immunoblotting with an iodinated OV-TL3 probe revealed two antigenic determinants with molecular weights of 20,000 and 40,000 daltons, respectively.

(b) An enzymatic analysis of the CA-TL3 antigen demonstrates that the antigen is only slightly sensitive to proteolytic digestion. Binding of OV-TL3 to OVCAR-3 cells (1) decreased slightly after treatment with pronase and trypsin, (2) did not change after papain treatment, and (3) increased after treatment with neuraminidase.

(c) The CA-TL3 antigen is not shed from ovarian cancer cells. Iodinated OV-TL3 was mixed with dilutions of serum and ascites samples from ovarian cancer patients. The mixtures were then added to wells containing ovarian cancer cells, and the inhibition of antibody binding was determined (see exemplification below). OV-TL3 binding was only slightly inhibited by non-diluted samples, which was the same

for serum samples from healthy donors or from ovarian cancer patients.

(d) The monoclonal antibodies OV-TL3 and OC125 recognize distinct antigenic determinants that are associated with human ovarian carcinomas, and the steric configuration does not hinder the antibodies when they are bound to the cells. Therefore, more antibody can be bound when both antibodies are incubated simultaneously with the ovarian cancer cells.

Of particular importance in distinguishing the antigen of the present invention from other antigens, including other ovarian tumor associated antigens, is the specificity of monoclonal antibody OV-TL3 for at least two determinants of the antigen characterized herein. Additionally, the specific reactivity of OV-TL3 for the antigen defined by the invention provides a means for isolation and purification of the antigen from other material of human origin, and ultimately the characterization of antigenic determinants. The CA-TL3 antigen can be used to construct an immunoadsorbent for purification of antibody reactive with the antigen by attaching the antigen to a solid phase.

The purified antigen and determinants thereof are useful in the production of monoclonal antibodies for diagnostic and therapeutic application using techniques well known in the art. For example, murine hybridomas producing monoclonal

antibodies may be obtained. The isolated immunoreactive 20 kDa or 40 kDa species of CA-TL3 can be used as an immunogen preparation to raise anti-CA-TL3 antibody by employing the standard techniques of Kohler and Milstein Nature, 256, 495-497 (1975). For example, a mouse or other suitable host is immunized with purified CA-TL3. Spleen cells are harvested and fused with suitable mouse myeloma cells. The resulting hybridomas can be selected for anti-CA-TL3 antibody production on the basis of specific reactivity with the isolated CA-TL3 antigen.

Monoclonal antibodies raised against CA-TL3 are useful for the diagnosis and treatment of ovarian carcinoma. These antibodies can be used for in vivo tumor imaging to localize primary or metastatic ovarian carcinoma. For example, immunoscintigraphy can be used for tumor imaging. An antibody or antibody fragment (antigen binding fragment) specific for CA-TL3 conjugated to a label which generates a signal detectable by external scintigraphy (e.g., a gamma emitting radioisotope) is administered to a patient. After sufficient time to allow the labeled antibody to localize at the tumor site (or sites), the signal generated by the label is detected by a photo-scanning device. The detected signal is then converted to an image of the tumor. This image makes is possible to locate the tumor in vivo and to devise an appropriate therapeutic strategy.

Antibody fragments, rather than whole antibody molecules, are generally preferred for use in tumor imaging. Because they are distributed more readily in the tissues than are entire antibody molecules, antibody fragments accumulate at the tumor(s) more rapidly. Thus an image can be obtained in less time than is possible using whole antibody. These fragments are also cleared more rapidly from tissues, resulting in a lower background signal. Haber et al., U.S. Patent No. 4,036,945; Goldenberg et al., U.S. Patent No. 4,331,647. The antigen binding fragments Fab and $F(ab')_2$ are preferred. The Fab fragment can be prepared by digestion of the whole immunoglobulin molecule with the enzyme papain; and the $F(ab')_2$ fragment by digestion of the whole immunoglobulin molecule with pepsin according to any of several well known protocols. In addition, fragments can also be prepared by recombinant DNA techniques.

The antibodies or antibody fragments can be labelled with radioisotopes such as Indium-111 and Technetium-99m. The gamma-emitters Indium-111 and Technetium-99m are preferred because these radiometals are detectable with a gamma camera and have favorable half-lives for imaging in vivo. Antibody can be labelled with Indium-111 or Technetium-99m via a conjugated metal chelator, such as DTPA (diethylenetriaminepentaacetic acid). Krejcarek et al., Biochem. Biophys. Res. Comm. 77:581, 1977; Khaw, B.A. et al. Science 209:295,

1980; Gansow et al., U.S. Patent No. 4,472,509; Hnatowich, U.S. Patent No. 4,479,930, the teachings of which are incorporated by reference herein. A preferred method of conjugating the antibody or fragment with DTPA is by the dicyclic anhydride method. See Hnatowich et al., Science 220:613-615, 1983.

Human tissue specimens (e.g., biopsy samples) can be tested for expression of the CA-TL3 antigen by using monoclonal antibodies to the former in an immunohistochemical technique, such as the immunoperoxidase staining procedure. Alternatively, immunofluorescent techniques can be used to examine human tissue specimens. In a typical protocol, slides containing cryostat sections of frozen, unfixed tissue biopsy samples or cytological smears are air dried, and then incubated with the CA-TL3 antigen preparation in a humidified chamber at room temperature. The slides are layered with a preparation of fluorescently labelled antibody directed against the monoclonal antibody. The staining pattern and intensities within the sample are determined by fluorescent light microscopy.

The monoclonal antibodies which specifically react with the CA-TL3 antigen can be used in at least two different forms of therapy for treatment of ovarian carcinoma. First, in passive immunotherapy, an anti-tumor amount of monoclonal antibody is administered in a physiologically acceptable vehicle (e.g., normal saline) to a patient afflicted

with ovarian carcinoma. Intact antibody is preferred for this purpose because effector functions attributable to the F_c portion are retained.

Second, monoclonal antibodies which specifically bind to the CA-TL3 antigen can be used as targeting agents to deliver anti-cancer agents selectively to tumor cells. Various pharmaceutical or cytotoxic agents can be covalently or noncovalently coupled to the antibodies. Examples of useful therapeutic agents include: radioactive compounds (e.g., isotopes of Boron and Rhenium); agents which bind DNA, such as alkylating agents or various antibodies (e.g., daunomycin, adriamycin, chlorambucil); anti-metabolites (e.g., methotrexate); and inhibitors of protein synthesis (e.g., diptheria toxin and toxic plant proteins).

In the methods discussed above for imaging and treating human ovarian carcinoma, the monoclonal antibody OV-TL3 can be administered in combination with the monoclonal antibody OC125. Because OV-TL3 and OC125 recognize distinct antigenic determinants, and the steric configuration does not hinder the antibodies when they are bound to the cells, more antibody can be bound when both antibodies are administered simultaneously.

The invention is illustrated further by the following example, which is not to be taken as limiting in any way.

Exemplification

Cell Lines

Ovarian cancer cell line OVCAR-3 (Hamilton, T.C., Young, R.C., Louie, K.G., Behrens, B.C., McKoy, W.M., Grotziner, K.R. and Ozols, R.F., Cancer Res. 44:5286-5290, 1984) was used because it can be grown in vitro as well as in vivo in nude mice. The cell line was grown in vitro in Eagle's minimal essential medium supplemented with 10% FCS (foetal calf serum), nonessential amino acids (0.1 mM), 2 mM l-glutamine, 1 mM sodium pyruvate, pencillin G (50 units/ml) and streptomycin (50 ug/ml).

Monoclonal Antibodies

The monoclonal antibodies OC125 and OV-TL3 have been described (Bast, R.C., Freeney, M., Lazarus, H., Nadler, L.K., Colvin, R.B. and Knapp, R.C., J. Clin. Invest. 68:1331-1337, 1981; Poels, L.G., van Megen, Y., Vooijs, G.P., Verheyen, R.N.M., Willemen, A., van Niekerk, C.C., Jap, P.H.K., Mungyer, G. and Kenemans, P. J.N.C.I. 75:731-791, 1986). Hybridomas were grown either in tissue culture or as acites in BALB/c mice. Both OC125 and OV-TL3 are of the mouse IgG-1 type. The antibodies were purified as follows: Mouse ascites of hybridoma tissue culture supernatant was filtered and adjusted to pH 8 with 1.0 M Tris-HCl before application onto a Protein A-Sepharose column. The unbound materials were removed from the column by washing with 0.1 M

Tris-HCl until no further change in absorbance at 280 nm was seen. The antibodies were then eluted with 0.1 M citrate (pH 3.5). Fractions containing the purified antibody were pooled, concentrated and dialysed against 0.9% NaCl solution. $(Fab')_2$ fragments were prepared as described elsewhere (Johnstone, A. and Thorpe, R. Immunochemistry in Practice, Blackwell Science Publications: 53-55, 1982). The protein concentration was determined by measuring the absorbance at 280 nm, assuming that 1 mg/ml antibody reads 1.4.

Radioiodination

Iodination of OC125 and OV-TL3 was performed according to the one-vial method (Haisma, H.J., Hilgers, J. and Zurawski, V.R. Jr. J. Nucl. Med., in press). Briefly, 290 ug of antibody in 1 ml 0.1 M borate buffer (pH 8.2) was mixed with 1 mCi ^{125}I in a vial previously coated with 1 ml iodogen (50 ug/ml). After 10 minutes of incubation at room temperature, a sample was taken to determine the amount of incorporated iodine. One milliliter of AG1-X8 resin (Bio Rad, Richmond, CA) previously mixed with PBS (phosphate buffered saline) containing 1% bovine serum albumin (BSA), was added to absorb unbound iodine. The reaction mixture was filtered through a 0.22 um filter to remove the resin and sterilize the product. After iodination, the immunoreactivity of the antibodies was determined according to Lindmo (Lindmo, J., Boven,

E. and Cuttitta, F., J. Imm. Methods 72:77-89, 1984). Increasing numbers of ovarian carcinoma cells were added to one concentration of labeled antibody. After incubation at 4°C for 4 hours, the cells were washed. The amount of bound antibody was determined using a gamma counter. The reciprocal of the cell concentration was plotted against total input divided by bound antibody. Immunoreactivity was computed from the intercept on the y-axis. OC125 was labeled at 1 x 10^6 cpm/pmol (0.4 uCi/uM) of active antibody and OV-TL3 was labeled at 0.7 x 10^6 cpm/pmol (0.3 uCi/uM) of active antibody.

Immunoblotting

OVCAR-3 cells were lysed in sample buffer before electrophoresis on a 7.5% or 10% acrylamide gel with a 3% acrylamide stacking gel (Laemmli, U.K., Nature 227:680-685, 1970). The sample buffer contained 0.1% SDS. However, to prevent the inactivation of the antigens, (a) the sample buffer did not contain sulfhydryl reducing agents and (b) the samples were not heated before they were loaded on the gel. After electrophoresis, the gel was blotted onto nitrocellulose according to the method of Towbin (Towbin, H., Stagheling, T. and Gordon, J. Proc. Natl. Ac. Sci. 769:4350-4354, 1979). The transfer was performed at 2.0 Ampere for 2 hours with PBS as the transfer buffer. After incubation with PBS 10% FCS for 30 minutes at room temperature, the nitrocellulose sheets were overlayed with

^{125}I-labeled OC125 or OV-TL3 (1 X 10^6 cpm/ml) for 2 hours. These sheets were then autoradiographed by exposure to x-ray film with the aid of a Kodak X-Omatic intensifying screen for 18-48 hours at -80°C.

Enzymatic Treatments

Cell monolayers were prepared by seeding 2 x 10^4 OVCAR-3 cells/well in 96-well flat bottom tissue culture plates. Before use, the cells were grown to 80-90% confluency, washed with PBS and fixed with 0.25% glutaraldehyde in PBS for 10 minutes at room temperature. The wells were then treated with 200 ug/ml pronase, trypsin, or chymotrypsin in PBS or neuraminidase in 0.1 M acetate buffer (pH 4.5). After incubation for 1 hour at 37°C the wells were washed three times with PBS 10% FCS and antibody was added at 10 ug/ml. After incubation the wells were washed once more, then peroxidase-labeled goat anti-mouse IgG (Boeringer, Mannheim) was added to the wells. Following additional incubation and washes, substrate (O-Phenylenediamine, 1 mg/ml in 0.1 M acetate pH 5.0) was added, and, after development of the color reaction, the OD 490 of individual wells was read in an ELISA (enzyme-linked immunosorbent assay) reader.

Binding Assay

Cell monolayers were prepared as for enzymatic treatments. To determine affinity constants, the

wells were incubated with 50 ul labeled antibody for 4 hours at 4°C in PBS 10% FCS containing 0.1% NaN3 to prevent shedding and modulation. After three washes with PBS 10% FCS, binding was determined by counting in a gamma counter. Association and dissociation experiments were performed on both live and fixed (0.25% glutaraldehyde for 10 minutes) cells at room temperature. For association experiments, monolayers were incubated with 50 ul labeled antibody (100,000 cpm) in PBS 10% FCS, for different time periods. For dissociation, monolayers were incubated for 1 hour with labeled antibody. After washing, antibody was added at 100 ug/ml. At different time intervals labeled antibody which remained bound to the cells was determined by gamma counting.

Inhibition Assay

OVCAR-3 cells were prepared as for the enzymatic treatments. The cells were fixed with glutaraldehyde and incubated for 60 minutes at room temperature with different concentrations of either OC125 or OV-TL3 mixed with ^{125}I labeled antibody or with undiluted or 5 fold diluted serum or ascites samples from patients with ovarian carcinoma, mixed with tracer OC125 or OV-TL3.

Localization in Human Ovarian Carcinoma Xenografts

Female athymic Swiss-nu/nu mice (Taconic Inc., Germantown, NY) 5 to 8 weeks old were given

subcutaneous injections of 2.5 x 10^7 OVCAR-3 cells in the right flank. After 3 weeks, when tumors were approximately 1 cm in diameter, the animals were given 1 ug ^{125}I-labeled OC125 of OV-TL3 (2-3 uCi) in the retro orbital vein. To block uptake of free iodine by the thyroid, mice received 0.1% saturated potassium iodide in the drinking water. At 24 and 120 hour after antibody administration, mice were sacrificed (3 mice for each time point per antibody) and the dissected tissues were weighed and analyzed by a gamma counter.

Results

Analysis of Antigens by Immunoblotting

Ovarian cancer cell lysates were run on SDS polyacrylamide gels and blotted on nitrocellulose. The nitrocellulose blots were then incubated with either ^{125}I-labeled OC125 or OV-TL3, washed, and autoradiographed. OC125 bound to a broad band with an apparent molecular weight exceeding 200,000 daltons, which hardly entered the 3% stacking gel. OV-TL3 reacted with two bands with apparent molecular weights of 20,000 and 40,000 daltons (Figure 1).

Enzymatic Analysis of Antigens

The binding of the ^{125}I-labeled antibodies to ovarian cancer cells treated with pronase, trypsin, or neuraminidase was determined. Binding of OC125 to OVCAR-3 cells decreased after treatment with

pronase, papain, and trypsin, but increased slightly after treatment with neuraminidase. Binding of OV-TL3 to OVCAR-3 cells decreased after treatment with pronase and trypsin; papain treatment had no effect and neuraminidase treatment increased binding of OV-TL3 to OVCAR-3 cells (Table I). A control antibody, 115D8 (Hilkens, J., Buys, F., Hilgers, J., Hageman, Ph., Calafat, J., Sonnenberg, A. and Van der Valk, M. Int. J. Cancer 34:197-206, 1984) reactive with carbohydrate determinants on OVCAR-3 cells, did not show reduced binding after pronase, trypsin, or papain. Binding of 115D8 was reduced after neuraminidase treatment.

Table I. The affect on antibody binding after pretreating OVCAR-3 cells with various enzymes.

antibody	enzyme (% inhibition)			
	pronase	papain	trypsin	neuraminidase
OC125	93[1]	78	50	10
OV-TL3	18	0	15	-20

[1] Numbers represent percentage of binding inhibition as compared with control conditions.

Quantitation of OC125 and OV-TL3 Binding to Ovarian Cancer Cell Lines

Affinity constants were estimated and the number of antigenic determinants was determined by measuring the binding of the ^{125}I-labeled antibodies to the OVCAR-3 cell line. From linear double-reciprocal plots (Figure 2), affinity constants and the number of antigenic determinants could be calculated (Masuho, Y., Zalutski, M., Knapp, R.C. and Bast, R.C., Jr. Cancer Res. 44:2813-2819, 1984). The affinity constant for OC125 was 0.9×10^9, while that for OV-TL3 was 1.6×10^9. The number of antigenic determinants per cell was 6.0×10^6 for OC125 and 0.6×10^6 for OV-TL3.

Association and Dissociation of OC125 and OV-TL3

Association of the two monoclonal antibodies was determined by measuring the amount of ^{125}I-labeled antibody bound to live cells and fixed cells after different incubation periods (Figure 3). Association of OC125 was very slow, reaching 50% binding in 125 minutes (Figure 3A); whereas OV-TL3 associated much faster, with 50% binding in 30 minutes (Figure 3B). There was no difference in the association rates for OV-TL3 between live and fixed cells. Dissociation of OC125 and OV-TL3 was measured by incubating live and fixed cells with ^{125}I-labeled antibody for 1 hour, washing the cells and adding non-radioactive antibody at 10 ug/ml. At intervals, the amount of antibody that remained

bound to the cells was determined (Figure 4). OC125 dissociated with a $T_{\frac{1}{2}}$ of 125 minutes from fixed cells and 85 minutes from live cells (Figure 4A). For OV-TL3 no difference in dissociation was noted between live and fixed cells (Figure 4B). However, antibody disappearance was biphasic with apparent $T_{\frac{1}{2}}$ s of 60 and 300 minutes.

Inhibition by Serum and Ascites Samples

^{125}I-labeled antibodies were mixed with dilutions of serum and ascites samples from ovarian cancer patients. The mixtures were then added to wells containing ovarian cancer cells, and the inhibition of antibody binding was determined after 60 minutes of incubation. Figure 5 shows the binding inhibition of OC125 and OV-TL3 for serum and ascites samples diluted 5 fold. As was expected, OC125 binding was inhibited by some of the serum samples and all the ascites samples. At this dilution, inhibition of binding correlated well with CA125 values of the samples. Inhibition by serum samples varied between 0% (400 CA125 U/ml) and 37% (3000 CA125 U/ml) and inhibition by ascites samples was between 75% (2000 CA125 U/ml) and 94% (13,000 CA125 U/ml). Undiluted ascites samples completely inhibited the binding of OC125. Undiluted serum samples inhibited between 0% (35 CA125 U/ml) and 20-90% (99-3000 CA125 U/ml) (data not shown). OV-TL3 binding was slightly inhibited by non-diluted samples, which was the same for serum samples from

healthy donors or from ovarian cancer patients (data not shown). OV-TL3 binding was not inhibited at five fold dilutions of the serum and ascites samples.

Cross Inhibition of Antibodies

Cross inhibition experiments were performed to see whether both antibodies recognized similar epitopes on molecules. Iodinated antibody was mixed with different concentrations of cold antibody, and binding to OVCAR-3 cells was determined (Figure 6). Binding of OC125 could be completely inhibited by 0.1 mg/ml cold OC125 but not by OV-TL3; similarly, OV-TL3 binding could be inhibited by cold OV-TL3 but not by OC125.

Localization in Human Ovarian Cancer Xenografts

To compare the distribution of OC125 or OV-TL3 in vivo, we injected athymic mice bearing OVCAR-3 xenografts with the ^{125}I-labeled antibodies. Triplicate animals were sacrificed at 24 hours and 120 hours after injection and their tissues were analyzed. Figure 7 indicates the uptake of the antibodies in the various tissues. No significant differences between the two antibodies were found in uptake in blood, marrow, intestine, fat, muscle and lung. At 24 hours after injection uptake of OC125 in liver and spleen was significantly higher than uptake of OV-TL3. This difference was less apparent at 120 hours after injection. Kidney uptake was

higher for OV-TL3 at 24 hours as well as at 120 hours after injection. Tumor uptake of OC125 and OV-TL3 was similar at 24 hours after injection and about 5 times higher than uptake in other tissues. At 120 hours after injection of the labeled antibody, OV-TL3 uptake in tumor was about 7 times higher than uptake in other tissues and about twice as high as OC125 uptake in tumor.

Discussion

The monoclonal antibodies OC125 and OV-TL3 recognize distinct antigenic determinants that are associated with human ovarian carcinomas, and both show similar distribution patterns on tissue sections of human organs and tumors. OC125 binds to more than 80% of nonmucinous ovarian carcinomas including tumors of serous, endometrioid, clear cell, and undifferentiated histology (Kabawat, S.E., Bast, R.C., Bhan, A.K., Welch, W.R., Knapp, R.C. and Colvin, R.B. Int. J. Gyn. Path. 2:275-285, 1983). Traces of antigen can be found in the mucosa of the fallopian tube, endometrium, and endocervix. Using a radioimmunometric assay, one can measure levels of CA125 in the serum and ascites of patients with ovarian cancer. CA125 antigen can also be found in human milk, normal cervical mucus, in the central airway of the lung, and seminal plasma (Davis, H.M., Zurawski, V.R., Bast, R.C and Klug, T.L. Cancer Res. 46:6143-6148, 1986).

OV-TL3 binds to ovarian carcinomas of serous, mucinous, endometrioid and clear cell histology. When tested for the presence of antigen of OV-TL3, the epithelial lining of a borderline malignant papilliferous serous cystadenoma reacted weakly, as did ovarian cysts and the epithelial lining of a few endocervical invaginations and nabothian cysts (Poels, L.G., van Megen, Y., Vooijs, G.P., Verheyen, R.N.M., Willemen, A., van Niekerk, C.C., Jap, P.H.K., Mungyer, G. and Kenemans, P. J.N.C.I. 75:781-791, 1986). Although several other antibodies to human ovarian carcinoma have been reported (Bast, R.C., Freeney, M. et al., supra; Mattes, J.M. et al., supra; Tabliabue, A. et al., supra; Tsuji, Y. et al., supra; Thor, A., et al., supra; Poels, L.G. et al., supra; Taylor-Papadimitriou, J. et al., supra), OC125 and OV-TL3 were selected for this study because of their specificity for these tumors and their lack of reactivity with other tissues. These two monoclonal antibodies were compared in terms of their interaction with ovarian carcinoma cells in an attempt to further characterize the antigens recognized by these antibodies. For this study the human ovarian cancer cell line OVCAR-3 was used because it can be grown in vitro as well as in vivo in nude mice. The interaction of the two antibodies with ovarian cancer cells was compared in both systems.

OC125 recognized determinants on molecules with apparent weights of more than 200,000 daltons, as

analyzed by immunoblotting. The broad shape of the band indicated that the antigen might be a glycoprotein with different degrees of glycosylation. Enzymatic studies showed that the antigen was sensitive to proteolytic digestion, indicating that the antigenic determinant is probably protein in nature. These results are in close agreement with those published by Davis et al. who found that the antigen had an apparent mass of 200,000 to 1000,000 dalton (Davis, H.M. et al., supra). These investigators showed that the buoyant density of the antigen is between 1.25 and 1.35 g/ml, which may indicate that the antigen is slightly glycosylated.

OV-TL3 recognized determinants on molecules with apparent weights of 20,000 and 40,000 dalton. Enzymatic studies revealed that the OV-TL3 antigen is only slightly sensitive to proteolytic digestion and that neuraminidase treatment enhances antigen expression. This could mean that the antigen is a glycoprotein and that the antigenic determinant is on the protein backbone.

Cross inhibition experiments on OVCAR-3 cells showed that binding of ^{125}I-labeled OC125 was not influenced by OV-TL3 binding and that ^{125}I-labeled OV-TL3 binding was not affected by OC125 binding. Upon incubation of the tracers with the same antibody, complete inhibition of binding could be achieved. These results indicate that OC125 and OV-TL3 recognize different antigenic determinants on OVCAR-3 cells, and that the steric configuration

does not hinder the antibodies when they are bound to the cells; i.e., more antibody can be bound when both antibodies are incubated simultaneously with the ovarian cancer cells. This might have implications for therapy because a higher dose of externally administered radioactivity could be delivered to tumor when the antibodies are combined in a treatment.

The affinity constants for OC125 and OV-TL3 were similar: 0.9×10^9 for OC125 and 1.6×10^9 for OV-TL3. The number of antigenic determinants per cell (OVCAR-3) was 6.0×10^6 for OC125 and 0.6×10^6 for OV-TL3. The rate of association of OC125 was very slow (50% binding after 125 minutes) compared with that of OV-TL3 (50% binding after 30 minutes). Dissociation rates for the two monoclonal antibodies also differed. OC125 dissociated with a $T_{\frac{1}{2}}$ of 125 minutes on fixed cells and 85 minutes on live cells, suggesting that active shedding (or modulation) process may be involved in the dissociation of this antibody from live cells. OV-TL3 showed no difference in dissociation rates from live or fixed cells; however, dissociation seemed to be biphasic, with apparent $T_{\frac{1}{2}}$ of 60 minutes and 300 minutes, suggesting that both low and high affinity binding sites might be present on the cell surface. Masuho et al. studied the interaction of OC125 using other ovarian cancer cell lines and found similar affinity constants and numbers of antigens per cell. Dissociation of OC125, however, was very slow in their

study ($T_{\frac{1}{2}}$ exceeded 23 hours). Since they did not include excess antibody after the initial incubation, rebinding of the dissociated antibodies could occur and their measurements do not reflect the dissociation rate. In this study the association rate for OC125 was slow compared with that reported by Masuho et al., who found 50% binding after 1 hour of incubation. (They used OVCA-433 cells, on which antigen might be more accessible than on the OVCAR-3 cells.)

Antibody binding of OC125 was inhibited by the serum or ascites samples from ovarian cancer patients. This was expected, since other studies have shown that such samples contain CA125 and that the level of antigen is an indicator of the presence and status of the disease. OV-TL3 binding was inhibited by neither of these samples nor by supernatants of ovarian cancer cells grown in tissue culture. Tissue homogenates were able to inhibit binding (data not shown). These results suggest that OC125 antigen is shed from ovarian cancer cells and is, therefore, found in the serum and ascites samples, whereas OV-TL3 antigen is not shed from ovarian cancer cells and is, therefore, not found in these samples. The biodistribution of the two antibodies was evaluated in nude mice bearing subcutaneous tumors of the OVCAR-3 cell line. Uptake in liver and spleen was higher for OC125 than for OV-TL3 at 24 hours after injection. Large amounts of CA125 antigen are shed from tumor cells and can be found

in the serum and ascites from OVCAR-3 growing mice. The radiolabeled OC125 can form complexes by binding to circulating antigen (Haisma et al., in preparation). It is likely that these complexes are cleared through liver and spleen, resulting in higher uptake in these organs. Kidney uptake was higher for OV-TL3. This could be due to reactivity of the antibody with structures in the kidney. Another explanation could be that OV-TL3 is dehalogenized faster than OC125 resulting in a faster iodine excretion. Although in vitro experiments showed that OV-TL3 binds to fewer determinants on OVCAR-3 cells than OC125, tumor uptake was similar at 24 hours after injection. This could be because of the higher association and lower dissociation rates of OV-TL3. This would also explain the higher tumor uptake at 120 hours after injection for OV-TL3. Shedding of CA125 antigen could also attribute to the decreased binding of OC125 at later time points. Because antigenic modulation and shedding may be one of the factors that limit effective serotherapy in vivo, it would be important to choose antigenic targets that do not have this property for use in cancer therapy (Miller, R.A. et al., Blood 58:78, 1981; Ritz, J. et al., J. Immunol. 125:1506-1514, 1980). Because of its histological reactivity with ovarian cancers, its fast association and slow dissociation from ovarian cancer cells, and the lack of shed antigen, OV-TL3

can be exploited for use in immuno(radio)therapy or immuno-radioscintigraphy of ovarian cancer.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more that routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A cell surface antigen associated with primary and metastatic human ovarian carcinoma of the serous, mucinous, endometrioid and clear cell types characterized by specific reactivity with the OV-TL3 monoclonal antibody.

2. An immunoadsorbent for specific adsorption of antibody reactive with CA-TL3 comprising a solid phase to which is bound the isolated cell surface antigen of Claim 1.

3. An immunogen composition for immunizing an individual against CA-TL3 antigen comprising isolated CA-TL3 antigen in a physiologically acceptable vehicle.

4. An immunogen composition of Claim 3, wherein the isolated CA-TL3 antigen is characterized by specific reactivity with the OV-TL3 monoclonal antibody.

5. A method for producing monoclonal antibodies for use in the detection, diagnosis and treatment of cancer in humans comprising:
   a. immunizing a mouse or other suitable host with purified CA-TL3 antigen;
   b. fusing spleen cells of said immunized mouse or other suitable host with suitable

mouse myeloma cells, thereby obtaining a mixture of hybrid cell lines;

c. culturing said hybrid cell lines in a suitable medium;

d. selecting and cloning hybrid cell lines producing an antibody having specific reactivity with the CA-TL3 antigen; and

e. recovering monoclonal antibody thus produced.

6. A method of detecting the presence of CA-TL3 antigen on the surface of human cells, comprising the steps of:

a. contacting the cells with labeled OV-TL3 antibody;

b. allowing the antibody to bind to the cells;

c. separating the cells from the unbound antibody; and

d. determining the label associated with the cells to determine the presence of CA-TL3 antigen on surface of the cells.

7. A method of detecting the presence of CA-TL3 antigen on the surface of human cells, comprising the steps of:

a. contacting the cells with OV-TL3 antibody;

b. allowing the antibody to bind to the cells;

c. separating the cells from the unbound antibody;

d. contacting the cells with a labeled second antibody which binds to OV-TL3;

e. separating the cells from unbound labeled second antibody; and

f. determining the label associated with the cells to determine the presence of CA-TL3 antigen on the surface of the cells.

8. A method of imaging ovarian carcinoma, comprising the steps of:

a. administering to an individual suspected of having an ovarian tumor a radiolabeled monoclonal antibody, or fragment thereof, which specifically reacts with the CA-TL3 antigen;

b. allowing the radiolabeled antibody or fragment to accumulate at a tumor site;

c. detecting the signal generated by the label by means of a photo-scanning device; and

d. converting the detected signal to an image of the tumor.

9. A method of Claim 8, wherein the monoclonal antibody is obtained by the method of Claim 5.

10. A method of Claim 8, wherein a $(Fab')_2$ or a Fab fragment is administered.

11. A method of Claim 8, wherein the radiolabel is Indium-111 or Technetium-99m.

12. A method of Claim 8, wherein the photo-scanning device is a gamma-camera.

13. A method of Claim 8, wherein a monoclonal antibody which specifically reacts with the CA125 antigen is administered in combination with a monoclonal antibody which specifically reacts with the CA-TL3 antigen.

14. A method of imaging ovarian carcinoma, comprising the steps of:

a. administering to an individual suspected of having an ovarian tumor an Indium-111 or Technetium-99m labeled monoclonal antibody fragment which specifically reacts with the CA-TL3 antigen;

b. allowing the radiolabeled antibody fragment to accumulate at a tumor site;

c. detecting the signal generated by the label by means of a gamma-camera; and

d. converting the detected signal to an image of the tumor.

15. A method of immunotherapy of an ovarian tumor comprising administering to an individual afflicted with the tumor an anti-tumor amount

of a monoclonal antibody which specifically reacts with the CA-TL3 antigen.

16. A method of Claim 15, wherein a monoclonal antibody which specifically reacts with the CA125 antigen is administered in combination with a monoclonal antibody which specifically reacts with the CA-TL3 antigen.

17. A method of Claim 15, wherein the monoclonal antibody is obtained by the method of Claim 5.

18. A method of treating ovarian carcinoma, comprising administering to an individual afflicted with an ovarian tumor an anti-tumor amount of a conjugate, the conjugate comprising:

a. a monoclonal antibody that specifically reacts with the CA-TL3 antigen; and

b. an anti-tumor agent.

19. A monoclonal antibody of Claim 18 which is obtained by the method of Claim 5.

20. A method of Claim 18, wherein the anti-tumor agent is a radioisotope, an anti-metabolite or a cytotoxin.

21. A method of Claim 18, further comprising the administering of a second conjugate to the

individual at the same time, the second conjugate comprising:

a. a monoclonal antibody that specifically reacts with the CA125 antigen; and

b. an anti-tumor agent.

22. A monoclonal antibody of Claim 21 which is obtained by the method of Claim 5.

23. A method of Claim 21, wherein the anti-tumor agent is a radioisotope, an anti-metabolite or a cytotoxin.

24. A method of diagnosing ovarian carcinoma, comprising the steps of:

a. contacting a tissue specimen from a patient suspected of being afflicted with ovarian carcinoma with an antibody which specifically reacts with the CA-TL3 antigen;

b. determining whether the antibody binds to cells of the tissue specimen by immunohistochemical techniques, the binding of the antibody being an indication of the presence of ovarian carcinoma.

25. A method of Claim 24, wherein the antibody is obtained by the method of Claim 5.

26. A method of Claim 24, wherein the immunohistochemical technique is the immunoperoxidase staining technique.

27. A method of Claim 24, wherein the immunohistochemical technique is the immunofluorescent technique.

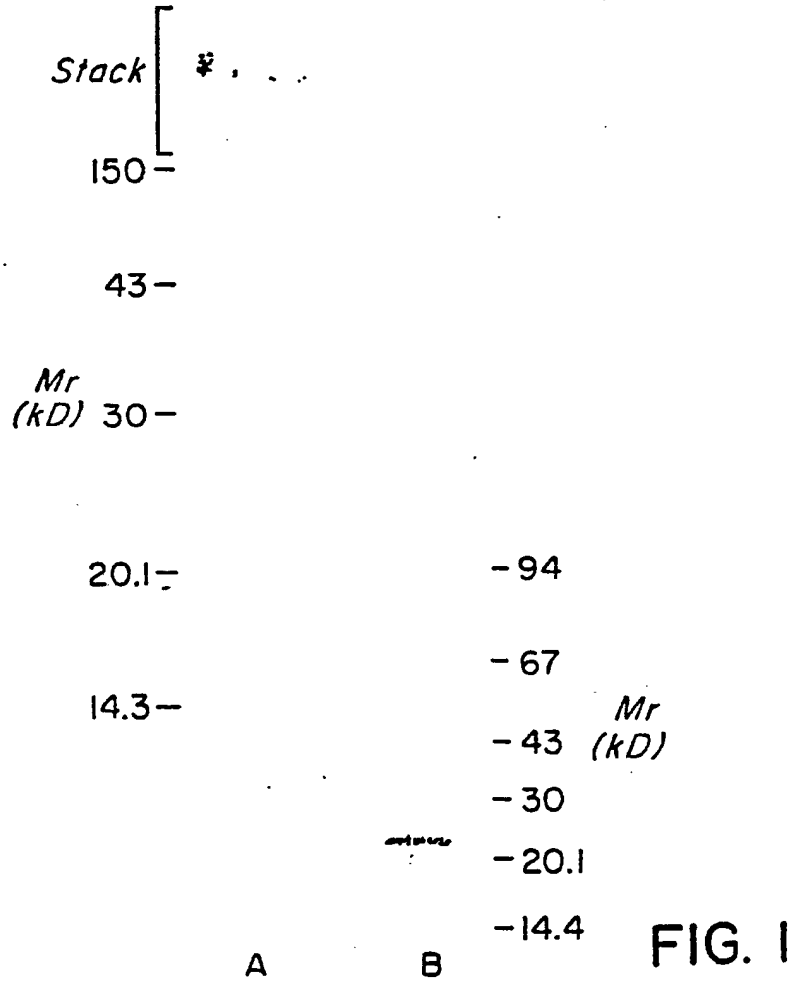

FIG. 1

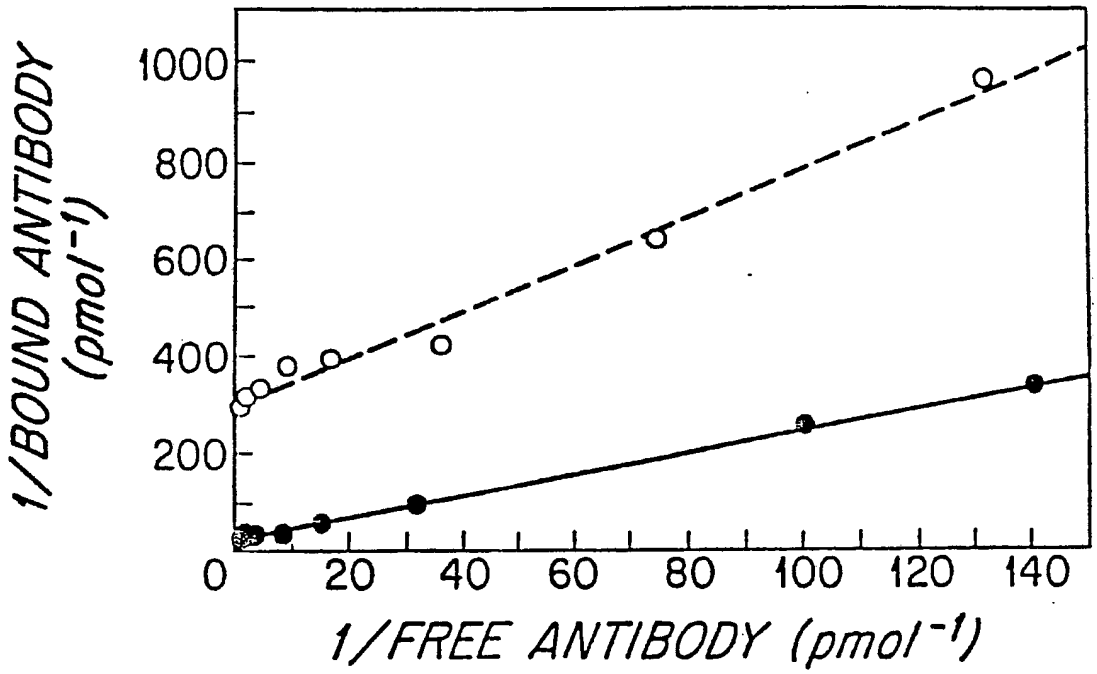
1/BOUND ANTIBODY (pmol⁻¹)
1000
800
600
400
200
0
20
40
60
80
100
120
140
1/FREE ANTIBODY (pmol⁻¹)

Figure 2

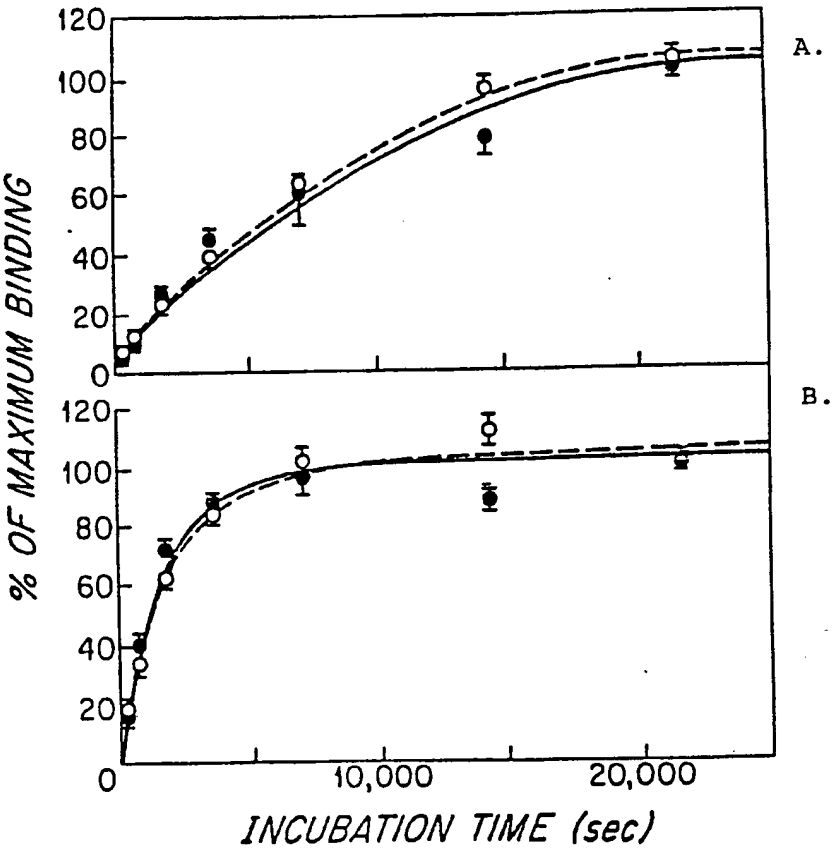
120
100
80
60
40
20
0
A.
B.
% OF MAXIMUM BINDING
10,000
20,000
INCUBATION TIME (sec)

Figure 3

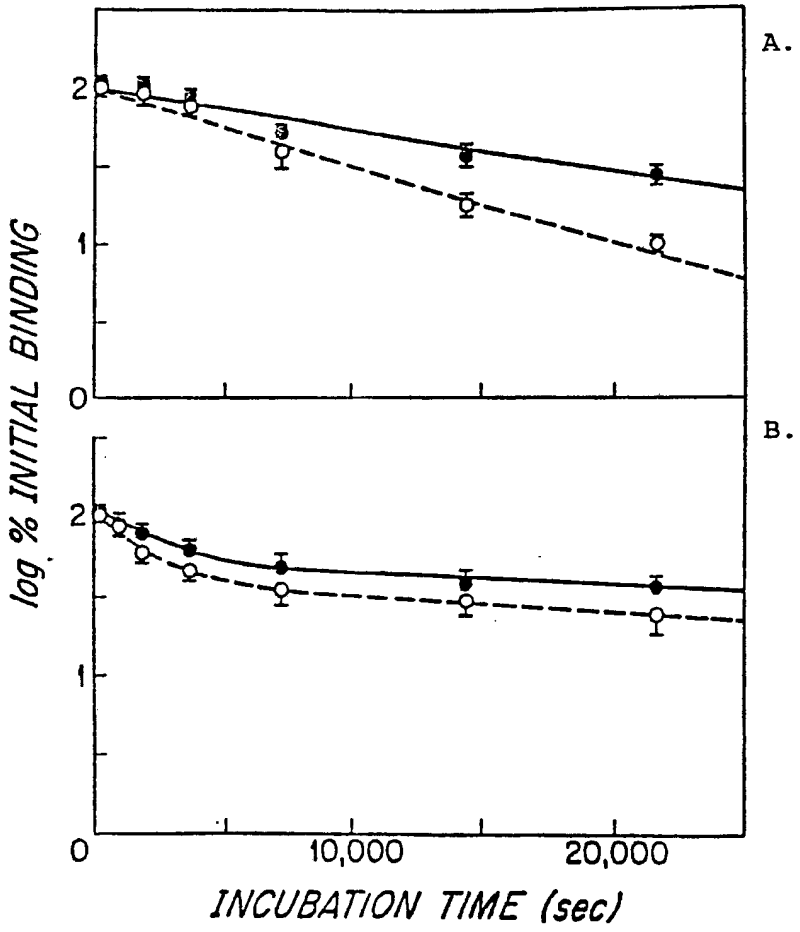
A.
B.
2
1
0
2
1
0
10,000
20,000
log % INITIAL BINDING
INCUBATION TIME (sec)

Figure 4

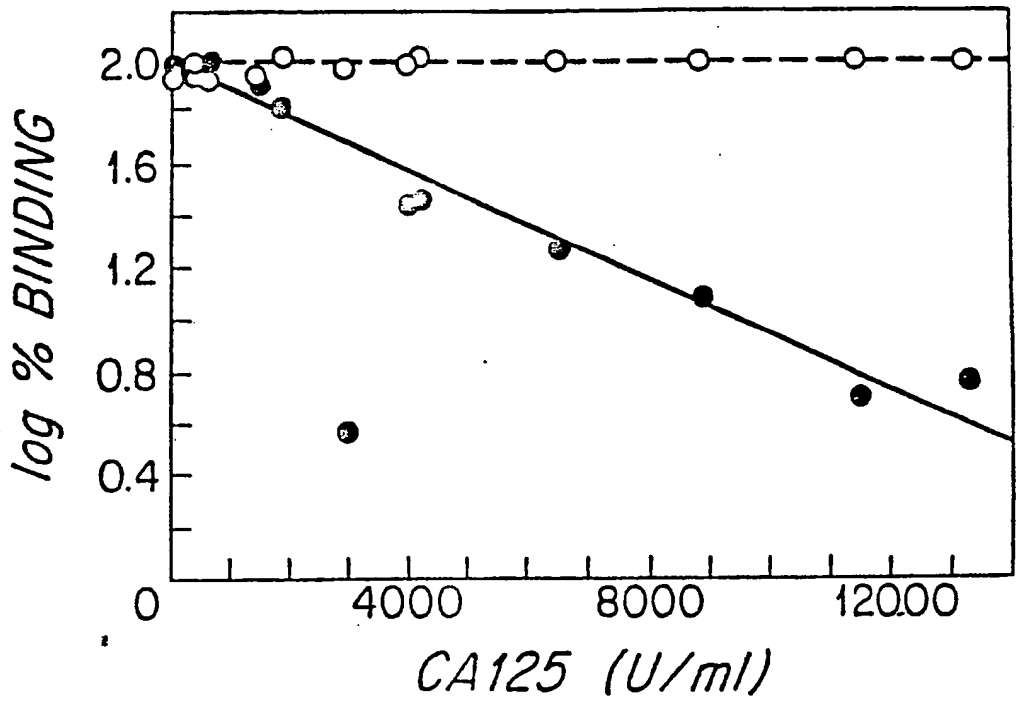

Figure 5

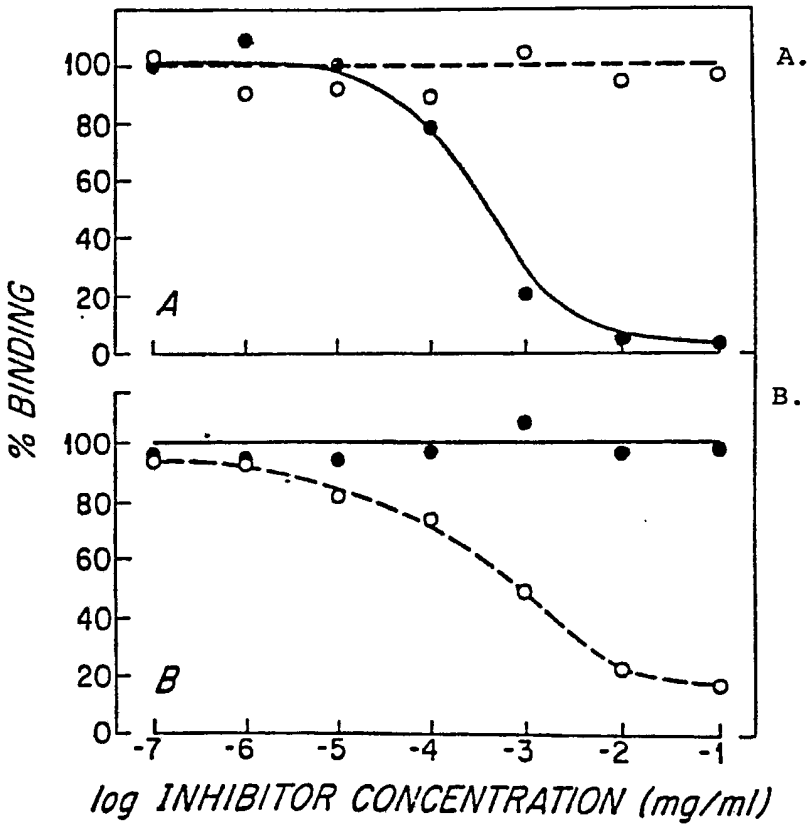
A.
B.
100
80
60
40
20
0
A
B
% BINDING
-7
-6
-5
-4
-3
-2
-1
log INHIBITOR CONCENTRATION (mg/ml)

Figure 6

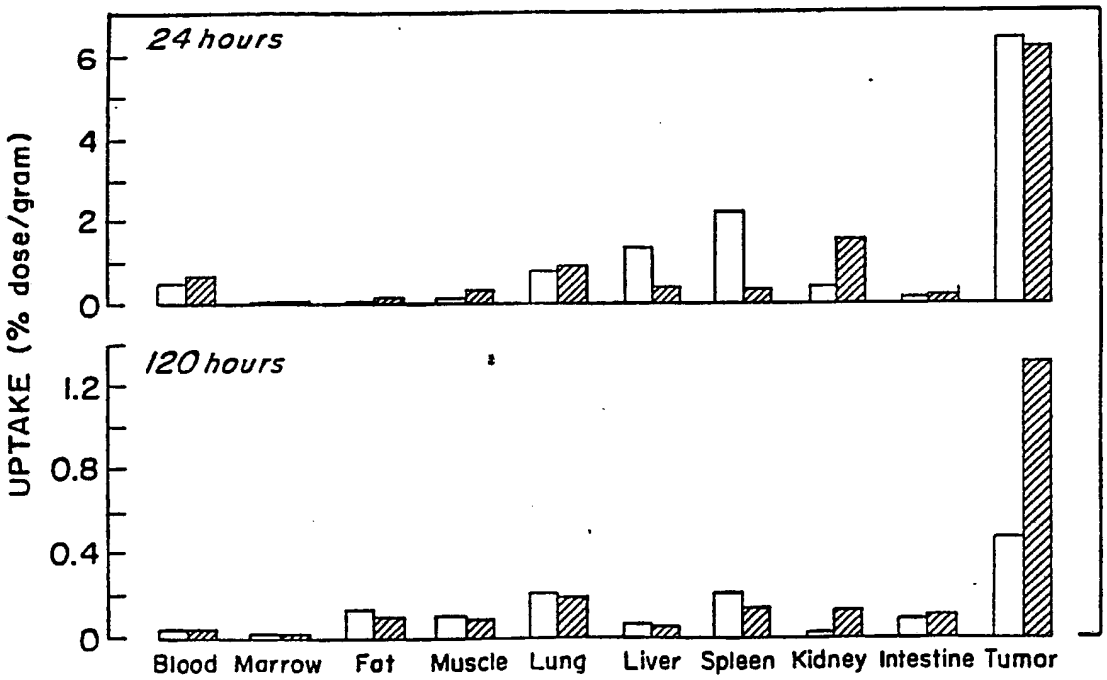
24 hours
120 hours
UPTAKE (% dose/gram)
6
4
2
0
1.2
0.8
0.4
0
Blood
Marrow
Fat
Muscle
Lung
Liver
Spleen
Kidney
Intestine
Tumor

FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/02831

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: G 01 N 33/574, A 61 K 39/395, A 61 K 49/00, C 12 P 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	G 01 N; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Journal National Cancer Institute, Vol. 76, No. 5, May 1986, L G Poels et al.: "Monoclonal Antibody Against Human Ovarian Tumor-Associated Antigens", pages 781-791 see page 783 --	1,6,24-27 2-5,7-17,19,22
Y	US, A, 4 472 371 (BURCHIEL S W et al.) 18 September 1984 see column 1 - column 4 and claim 33 --	1-12, 14
Y	EP, A, 0 226 418 (CETUS CORPORATION) 24 June 1987 see the whole document and corresponding A3 document --	19, 22
Y	EP, A, 0 226 419 (CETUS CORPORATION) 24 June 1987 see the whole document --	19, 22

./.

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21st December 1988	20 JAN 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Dialog Information Services, File 351: World Patent Information, accession No. 4524157, Pastan I et al.: "Monoclonal antibody against ovarian cancer cells conjugated to Pseudomonas exotoxin to produce an immuno-toxin for chemotherapeutic treatment of ovarian cancer", & US A 6888960, 7 April 1987 see the whole document	19, 22
Y	Cancer Research Vol. 45, pages 379-385, January 1985, E. Tagliabue et al.: "Generation of Monoclonal Antibodies Reacting with Human Epithelial Ovarian Cancer", see page 380, left column, paragraphs 4 and 5	24-27
A	Journal National Cancer Institute, Vol. 76, No. 6, June 1986, pages 995-1005, A. Thor et al.: "Tumor-Associated Glycoprotein (TAG-72) in Ovarian Carcinomas Defined by Monoclonal Antibody B72.3", see table 1	1-6, 24-27
A	Dialog Information Services, File 351: World Patent Information, accession no. 4000925, Tanabe Seiyaku KK, "Muoinio ovary tumour cell monoclonal antibody produced by hybridoma produced by fusion of myeloma cell", & JP A 60231622, 18 November 1985 see the whole document	19,22

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers \18,20,21,23/, because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv)

Method for treatment of the human or animal body by means of surgery or therapy, as well as diagnostic methods.

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

PCT/US88/02831

SA 24504

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 02/11/88
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4472371	18-09-84	EP-A- 0028092	06-05-81
		JP-A-56095119	01-08-81
		US-A- 4311688	19-01-82
		CA-A- 1152431	23-08-83
		US-A- 4478815	23-10-84
		US-E- 32417	12-05-87
EP-A- 0226418	24-06-87	JP-A-62209098	14-09-87
EP-A- 0226419	24-06-87	JP-A-62215600	22-09-87

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